Biomimetic Hydroxyapatite–Drug Nanocrystals as Potential Bone Substitutes with Antitumor Drug Delivery Properties**

By Barbara Palazzo, Michele Iafisco, Mariarita Laforgia, Nicola Margiotta, Giovanni Natile, Claudia L. Bianchi, Dominic Walsh, Stephen Mann, and Norberto Roveri*

This Full Paper investigates the adsorption and desorption of the anticancer drugs cis-diamminedichloroplatinum(II) (CDDP, cisplatin) and the new platinum(II) complex di(ethylenediamineplatinum)medronate (DPM), as well as the clinically relevant bisphosphonate alendronate, towards two biomimetic synthetic HA nanocrystalline materials with either plate-shaped (HAs) or needle-shaped (HAns) morphologies and different chemico-physical properties. The adsorption and desorption kinetics are dependent on the specific properties of the drugs and the morphology of the HA nanoparticles. Adsorption of the platinum complexes occurs with retention of the nitrogen ligands but the chloride ligands of cisplatin are displaced. Despite their opposite charges, the negatively charged alendronate bisphosphonate and the positively charged aqated cisplatin are strongly adsorbed, while the neutral DPM complex shows lower affinity towards the negatively charged apatitic surface. The data suggest that adsorption of the two platinum complexes is driven by electrostatic attractions, while interaction between the alendronate and the HA surface takes place by ligand exchange in which the two phosphonate groups of the drug molecule replace two surface phosphate groups. Significantly, adsorption of positively charged hydrolysis species of cisplatin is more favored on the phosphate-rich HAns surface while adsorption of negatively charged alendronate is more favored on the calcium-rich HAs surface. The latter type of short-range electrostatic interactions also appear to dominate the desorption kinetics; consequently, drug release is greater for neutral DPM than for charged alendronate and aqated cisplatin. Moreover, while the release per unit area of charged species is the same for the two types of HAS, the release of DPM is faster from HAns, which is lower in surface calcium, than for HAs. Overall, this work demonstrates that the properties of HA nanocrystals can be modulated in such a way to produce HA/biomolecule conjugates tailored for specific therapeutic applications.

1. Introduction

Calcium phosphates are physiological constituents of bones and are used to produce biomaterials for bone repair.[1] However, bioinert calcium phosphates, which have no influence on the surrounding living tissue,[2,3] are increasingly being substituted by bioactive calcium phosphates that can interact with the surrounding bone tissue and promote faster formation of new bone. The chemical and biological properties of the latter are strictly linked to their nanoscale dimensions, which require a nanoscience approach. Moreover, as the mineral phase of bone is constituted of carbonated hydroxyapatite (HA) crystals with a length of about 100 nm, width of 20–30 nm, and thickness of 3–6 nm, biomimetic calcium phosphates need to be synthesized with similar nanoscale dimensions, as well as with properties such as low crystallinity, nonstoichiometric composition, crystalline disorder, and the presence of carbonate ions in the crystal lattice. Indeed, the excellent biological properties of HA, such as nontoxicity, lack of inflammatory and immunitary responses, and high bio-resorbability can be significantly increased by lowering the crystallinity of synthetic HA.[4] Furthermore, the surface functionalization of HA nanocrystals with bioactive molecules makes them able to transfer information to and act selectively on the biological environment, and this represents a main challenge for innovative bone substitute materials. In this context, the synthesis of apatite nanocrystals loaded with antitumor drugs that can be released by a controlled kinetic process represents an attractive goal. For example, the application of such a material to the chemotherapeutic treatments of osteosarcoma could result in tumor inhibition accompanied by low levels of systemic toxicity.[5–8]
For many years, cis-diaminedichloridoplatinum(II) (cisplatin; CDDP) has been widely utilized as an anticancer drug despite its nephrotoxicity and other side effects observed for high-dose treatment; indeed, it is one of the most active antitumor agents in the treatment of osteosarcoma. The adsorption and release of cisplatin by slurries of synthetic HA crystals in aqueous media has been investigated as a function of ionic composition and the data indicated that chloride ions reverse the hydrolysis and that acid dissociation of the aquated species favors binding to calcium phosphate. Moreover, investigations carried out using either poorly or well crystallized carbonated HA have shown that cisplatin adsorption increases with decreasing HA crystallinity and that the less crystalline the material, the slower the drug release. This result is particularly interesting because the poorly crystalline apatite examined exhibits a surface area, morphology, crystallite size and Ca/P molar ratio close to those observed for bone apatite crystals.

Calcium deficient apatites have also been investigated as platforms for the specific delivery to bone of ginal bisphosphonates (BPs), which are a class of drugs developed for use in various diseases of bone, tooth, and calcium metabolism. Bisphosphonates (BPs) are characterized by two phosphonate groups attached to a single carbon atom and have strong affinity for Ca ions of bone apatite. This property is the basis for their use as inhibitors of ectopic calcification and of bone resorption. In general, the physicochemical effects of BPs include binding to crystals of calcium phosphate and inhibition of their growth, aggregation, and dissolution. Some BPs, such as alendronate, have already been approved for clinical use in Paget’s disease, hypercalcemia of malignancy, and osteoporosis. Significantly, the basic P–C–P structure of BPs can be widely modified by changing the substituents at the central carbon atom. These changes are likely to influence the behavior of BPs in the biochemical processes. However, studies of the solid/liquid interfacial properties of HA/BPs conjugates have shown that differences among the bisphosphonates may also influence their mechanisms for binding and inhibiting crystal growth and dissolution. Thus differences among potent bisphosphonates, such as the apparently more prolonged duration of action of alendronate and zoledronate compared with the more readily reversible effects of etidronate and risedronate, can be explained in part by their ability to absorb onto bone minerals.

Although the mechanisms for binding of cisplatin and alendronate onto different HA surfaces has been separately investigated, a comparative study of their interaction onto apatitic nanocrystals synthesised with variable surface areas and crystal dimensions within the range of biomimetic parameters, has not been undertaken even though a detailed solid/surface characterization of these systems would provide important knowledge for designing new and more effective treatment systems. Moreover, platinum(II) compounds with aminophosphonic acids have also been proposed as a means for targeting a cytotoxic moiety to the bone surface. These compounds exhibit antimetastatic activity and reduce bone tumor volume, and are less nephrotoxic than cisplatin. To our knowledge, experimental investigations concerning Pt(II)-aminophosphonic acid interactions with HA nanocrystals have not yet been undertaken.

The aim of the present study is to develop new biomimetic apatite nanocrystals for potential use in bone implantation, which in addition function as a local targeted delivery system for anticancer and antimetastatic drugs with controlled release properties. As already pointed out, the adsorption and release of bioactive molecules are strongly affected not only by the chemical properties of the drug molecule but also by the chemical and structural characteristics of the HA substrates. Thus, in this paper we have investigated the adsorption and release of cisplatin (Fig. 1a), alendronate (Fig. 1b), and di(ethylenediamineplatinum)medronate (DPM) (Fig. 1c) using two biomimetic synthetic hydroxyapatite nanocrystal materials with either plate-shaped (HAp) or needle-shaped (HAn) morphologies and with different physicochemical surface properties. Cisplatin and DPM are comparable in water solubility (1–2 mg mL⁻¹ and 0.9 mg mL⁻¹ at room temperature, respectively), while alendronate is much more soluble (10 mg mL⁻¹ at room temperature). The pKₐ values for the alendronate phosphate groups are: pKₐ₁ = 1.35; pKₐ₂ = 2.87; pKₐ₃ = 7.03; and pKₐ₄ = 11.3, whereas no pKₐ values are reported for the platinum complexes because metal ion coordination results in loss of phosphonate basicity. These bioactive molecules were chosen in order to compare the behavior of metal based drugs (CDDP and DPM) with that of a classical organic drug (alendronate), and evaluate the effect of the overall charge of the drug molecule on influencing the drug affinity for apatite nanocrystals with variable structural and chemical properties.

Figure 1. Molecular structure of a) cis-diaminedichloridoplatinum(II) (cisplatin, CDDP), b) alendronate (the overall negative charge is neutralized by different cations), and c) di(ethylenediamineplatinum)medronate (DPM).

2. Results

2.1. Synthesis and Characterization of the HA Nanocrystals Used as Drug Carriers

HA nanocrystals with nearly stoichiometric bulk Ca/P ratio (1.67, inductively coupled plasma (ICP) analysis) were synthesized according to two different procedures to produce plate- or needle-shaped bonelike apatite phases with different physical properties (Table 1). The surface Ca/P ratios determined by X-ray photoemission spectroscopy (XPS) analyses were significantly lower, indicating calcium deficiency as a result of surface disorder. In addition, although the bulk Ca/P ratios were similar, the surface Ca/P ratio was lower for the HAn nanocrystals.
compared with the HAp particles, suggesting that the former were more surface deficient in calcium ions.

Transmission electron microscopy (TEM) revealed the presence of clusters of platelike (length 25 ± 5 nm) or needlelike (length 100 ± 20 nm) nanoparticles for the HAp and HA nanoparticles, respectively (Fig. 2a and b). The specific surface area of the HAp nanocrystals was slightly larger than that determined for the HA nanoparticles (Table 1).

Table 1. Physicochemical characteristics of the two different types of HA nanocrystals used as drug carriers. Values are mean ± SD.

<table>
<thead>
<tr>
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<th>HA nanoparticles (HAp)</th>
<th>HA nanoparticles (HAp)</th>
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<tbody>
<tr>
<td>Degree of crystallinity [%]</td>
<td>52 ± 8</td>
<td>30 ± 4</td>
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<tr>
<td>Domain size along the c direction [Å]</td>
<td>350 ± 15</td>
<td>230 ± 10</td>
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<tr>
<td>Bulk Ca/P molar ratio</td>
<td>1.65 ± 0.1</td>
<td>1.62 ± 0.1</td>
</tr>
<tr>
<td>Surface Ca/P atomic ratio</td>
<td>1.30 ± 0.05</td>
<td>1.45 ± 0.05</td>
</tr>
<tr>
<td>Specific surface area [m² g⁻¹]</td>
<td>100 ± 5</td>
<td>120 ± 6</td>
</tr>
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Figure 2. TEM images of synthetic HA nanocrystals with a) plate-shaped morphology (HAp) (scale bar = 100 nm), and b) needle-shaped morphology (HA nanoparticles) (scale bar = 200 nm).

The crystal domain sizes along the c direction were calculated by Scherrer’s formula (see Experimental section) using the 2θ = 26° (002) diffraction peak, and were 350 and 230 Å for HAp and HAn nanoparticles, respectively. The HAp diffraction pattern was very similar to that recorded for deproteinized bone apatite (Fig. 3c), and this was quantitatively confirmed by the similarity of the two crystal domain sizes (230 and 213 Å, respectively).

2.2. Adsorption of Drug Molecules by HA Nanocrystals

The above differences in morphological and surface properties of the HAp and HA nanoparticles were investigated with respect to the adsorption and release of various drug molecules. The adsorption profiles of cisplatin (CDDP), alendronate, and di(ethylenediamineplatinum)medronate (DPM) using aqueous solutions containing an initial drug concentration of ca. 10⁻³ M in the presence of HAp or HA nanoparticles are shown in Figure 4. The plots show that the maximum uptake of cisplatin or DPM was reached after about 190 h for both types of HA nanocrystals, whilst the uptake of alendronate was significantly faster reaching a maximum value after about 24 h. In each case, slightly higher drug affinities were observed in the presence of HAp nanoparticles compared with HA nanoparticles. Typically, the percentage of drug adsorbed onto the HAp nanoparticles with respect to the initial drug concentration was 100, 89, and 68 % for alendronate, cisplatin, and DPM, respectively, which corresponded to a HA loading level of 5.6, 5.8, and 7.6 wt % of adsorbed drug (100 = Wdrug-loaded HA).

In contrast, the drug percentage adsorbed onto HA nanoparticles was 92 %, 82 %, and 52 %, for alendronate, cisplatin, and DPM, respectively (corresponding to HA loading levels of 5.2 %, 5.4 %, and 6.3 %). The higher level of alendronate affinity for HAp nanoparticles than for HA nanoparticles was more pronounced in concentrated alendronate solutions (4.6 x 10⁻³ M), where the percentage of drug adsorbed onto HAp and HA nanoparticles was 64 % and 39 % (corresponding to 12.6 % and 7.9 % loading levels), respectively.

Although for both HAp and HA nanoparticles, the bulk Ca/P ratios were very similar for both the drug-loaded and the unloaded materials (ICP analysis), the surface Ca/P ratios determined by XPS analyses were decreased for the HA–drug conjugates compared to the unloaded HA nanoparticles, consistent with adsorption of the drug molecules specifically onto the nanocrystal surfaces. The decrease in Ca/P ratio between a 5.6 % alendronate-loaded HAp conjugate and an unloaded HAp sample was 0.34 % (1.11 and 1.45, respectively), which were greater than the corresponding value of 0.19 % observed for 5.2 % alendronate-loaded HA nanoparticles and unloaded HA nanoparticles (1.11 and 1.30, respectively). A similar trend was observed for the DPM-loaded HA samples, which showed Ca/P ratios of 1.15 and 1.45 for DPM-loaded HAp (7.6 %) and unloaded HAp nanoparticles, compared with corresponding ratios of 1.26 and 1.30 for...
DPM-loaded (6.3%) HAns and unloaded HAns. Significantly, no appreciable release of phosphate from the HA nanocrystal surfaces was observed during cisplatin or DPM adsorption. In contrast, $2 \pm 0.5$ phosphate moles were released for every mole of alendronate adsorbed onto the HA nanoparticles, suggesting that this process occurred by ligand exchange of two surface phosphate anions with two phosphonate groups of the adsorbed drug molecule.

Figure 3. XRD patterns of a) bone HA, b) HAp, and c) HA.

Figure 4. Adsorbed mass percentages from $10^{-3}$ M solutions of cisplatin (CDDP, circles), DPM (squares), or alendronate (triangles), and from a $4.6 \times 10^{-3}$ M solution of alendronate (thick lines) as a function of time for plate-shaped (full symbols) and needle-shaped (empty symbols) HA nanocrystals. Cisplatin and DPM concentrations were determined by ICP-OES analyses, alendronate concentrations by UV-vis spectroscopy. Data represent mean values and error bars indicate the associated standard deviations.
Binding of the phosphonate groups of alendronate onto the HA nanoparticles was investigated by analysis of the spectral features of the O 1s and P 2p XPS peaks (Fig. 5). An intense band at 530.68 eV was observed for the alendronate-loaded HAns conjugate (Fig. 5c), which was considered as a convolution of the alendronate band at 530.37 eV (Fig. 5b), the HAns band at 531.36 eV (Fig. 5a), and significantly, a band at 531.68 eV originating from alendronate molecules adsorbed onto the HA surface. The P region of the alendronate-loaded HA XPS spectra (data not shown) indicated that the binding energy observed at 133.0 eV was due to the presence of both the HAns phosphate (133.5 eV) and alendronate phosphonate (132.3 eV).

XPS analyses were also undertaken on cisplatin-loaded HA samples. The Pt/N ratios were 0.50 ± 0.05 and 0.60 ± 0.05 for cisplatin-loaded HAns and HAps, respectively. These data were close to the stoichiometric values for cisplatin, revealing that the platinum complex does not undergo Pt–N bond degradation during adsorption onto the HA nanocrystals. On the other hand, no chlorine was detected by XPS analyses, clearly indicating that cisplatin undergoes hydrolysis before deposition onto the HA surface.[14,15] The oxygen region of the spectrum showed three peaks at 531.0, 533.1 and 534.1 eV, and as cisplatin alone has no oxygen signature (spectrum not shown), the decrease of the first oxygen-peak binding energy from a value of 531.36 eV in the unmodified HAns, was attributed to strong interactions between cisplatin and the HA surface. The same behavior was observed for the P peak at 133.0 eV, which was 0.5 eV lower than that observed for pure HA.

Similar XPS results were obtained for DPM-loaded HA conjugates. The Pt/N ratios were 0.40 ± 0.05 and 0.60 ± 0.05 for DPM-loaded HAns and HAps materials, respectively. These values were close to those for free DPM indicating that, like cisplatin, no Pt–N bond degradation occurred during surface adsorption. However, the P 2p binding energy for the DPM-loaded HA nanocrystals was 132.8 eV, which was lower than those observed for pure HA and cisplatin-loaded HA, indicating that the interaction between HA and DPM was stronger than that observed for cisplatin.

2.3. Drug Release Profiles

The release kinetics of alendronate, cisplatin, and DPM from HApS and HAns as a function of contact time in aqueous solution are plotted in Figure 6. UV-vis spectroscopic analysis indicated that the aqueous species released from cisplatin- or DPM-loaded HA nanoparticles had an adsorption peak maximum at 226 nm, which was not present in the blank solution (HEPES buffer). This band differed from the adsorption bands characteristic of the two platinum complexes, but nevertheless was ascribed to the release of the same platinum complex as confirmed by ICP-optical emission spectroscopy (OES) deter-
minations (see materials and methods). The amount of drug released from cisplatin- or alendronate-loaded HAns was, 45 and 21 % of the amount adsorbed, respectively, which were slightly lower than the corresponding values determined for cisplatin- and alendronate-loaded HAp samples (55 and 33 %, respectively). In the case of DPM-loaded HA nanoparticles, the release was faster and nearly quantitative, reaching equilibrium concentrations of 85 and 100 % for HA nanoparticles and HAp, respectively (Fig. 6).

3. Discussion

Adsorption and desorption phenomena depend on the physicochemical properties of the drugs and of the HA nanoparticles used. Adsorption is favored for alendronate and cisplatin, and less so for DPM (Fig. 4). For cisplatin, XPS analysis reveals the absence of chlorine, indicating hydrolysis, probably to a positively charged aqua species prior to surface adsorption, in agreement with previous studies. DPM can also undergo partial hydrolysis in solution but this takes place without complete displacement of the medronate group, so that overall the molecule remains neutral. Thus difference in charge of the hydrolyzed cisplatin and DPM complexes could account for the observed adsorption curves, particularly as the HA surface has a net-negative charge. The high adsorption of alendronate, however, despite its negative charge, suggests a different adsorption mechanism, and as two moles of phosphate are released per mole of adsorbed alendronate, the results suggest that adsorption of the bisphosphonate proceeds via ligand exchange. Indeed, it seems feasible that the ligand exchange mechanism of alendronate adsorption might also be facilitated by electrostatic interactions between the cationic aminopropyl side chain and negatively charged HA surface, which is consistent with recent studies by Nancollas et al.

For the three bioactive molecules investigated, the adsorption phenomena are also expected to be affected by the physicochemical properties and morphological characteristics of the two types of synthetic HA nanocrystals used as absorbent substrates. One such parameter affecting the drug binding is HA surface area, and in order to aid evaluation of the influence of degree of HA crystallinity and surface composition, normalization of the adsorption profiles with respect to surface area is shown in Figure 7. The normalized adsorption curves for HA nanoparticles and HAns are almost coincident for DPM adsorption ( ′ values give a confidence interval of 90 %), indicating that apart from the surface area, other physicochemical properties of the different HAs do not strongly affect the adsorption of this molecule at low concentrations. In contrast, normalized cisplatin and alendronate adsorption curves are close but not coincident for the two HAs, showing a slightly greater amount of adsorbed drug in the case of HAns as compared to HA nanoparticles. As HAns has a lower surface Ca/P ratio than HA nanoparticles, the surface charge is more negative, and this will favor adsorption of positively charged hydrolysis products of cisplatin and easier exchange of phosphate anions with alendronate phosphate groups. The difference in surface Ca/P ratios also appears to influence the adsorption of alendronate from more concentrated solutions (4.6 × 10^{-3} M) as the normalized profile for HAns falls below that for HA nanoparticles under these conditions. This could be as a result

Figure 7. Adsorbed mass percentages normalized with respect to HA surface areas for 10^{-3} M solutions of cisplatin (CDDM, circles), DPM (squares), and alendronate (triangles), and for 4.6 × 10^{-3} M solutions of alendronate (marked triangles) as a function of time for plate-shaped (full symbols) and needle-shaped (empty symbols) HA nanoparticles. Data represent mean values divided by a normalization constant; error bars indicate the associated standard deviations.
of the higher surface Ca/P ratio of the latter that favors adsorption of alendronate, which has high affinity for calcium. Thus, in more concentrated solutions, where HA binding sites are probably saturated, alendronate adsorption appears to be modulated by the surface Ca/P ratios, with increased adsorption on calcium-rich surfaces. In contrast, positively charged cisplatin adsorption is greater on phosphate-richer surface, and adsorption of neutral DPM molecules is not appreciably affected by differences in Ca/P ratios of the HA surfaces.

The release profiles of the drug molecules from the HA nanoparticles surfaces follow a different trend (DPM > cisplatin > alendronate) when compared with the adsorption process. UV analysis shows that the released species is the same for cisplatin and for DPM-loaded HAs, indicating that release of platinum from the latter probably occurs through complete breakage of the platinum–medronate bond. Therefore, the desorption process can be expected to be quite fast for DPM; slower for cisplatin, as the positively charged hydrolyzed cisplatin is linked quite strongly by electrostatic interactions to the negatively charged HA surface; and very slow for alendronate because of strong electrostatic interactions between the bisphosphonate groups and surface Ca\(^{2+}\) ions. We applied the diffusion-based model described by Higuchi\(^{[32]}\) to our release profiles (data not shown) but were unable to fit the experimental data, suggesting a non-diffusive mechanism for the systems under investigation. Normalized plots of the release profiles are almost coincident for cisplatin and alendronate (Fig. 8), indicating that desorption of these charged drugs is not affected by the nanocrystal surface composition (Ca/P ratio) but only by surface area.

In contrast, surface-normalized DPM-desorption curves (Fig. 8) reveal an increase in the amount of desorbed drug in the case of HAns as compared to HAp nanoparticles. If we assume that the release of this molecule occurs through complete cleavage of the Pt-phosphonate bond, we expect that this process will depend on a physicochemical property such as differences in the surface stoichiometry (Ca/P ratio) of the HA nanoparticles surfaces.

4. Conclusions

The in vitro adsorption and desorption of cisplatin, DPM, and alendronate towards needle-shaped or plate-shaped HA nanocrystals have been investigated. The HA surface area and surface charge (Ca/P ratio), as well as the charge on the adsorbed molecules and their mode of interaction with the HA surface, influence the adsorption and release kinetics of the three drugs investigated. Electrostatic binding of a cationic species of hydrolyzed cisplatin species to the negatively charged HA surface is greatly favored over the adsorption of uncharged DPM. On the other hand, binding of negatively charged alendronate to the HA nanoparticles surface is not curtailed because ligand exchange between the bisphosphonate group and two surface phosphate anions promotes strong adsorption. The above processes are modulated to some extent by the surface composition; for example, HAp nanoparticles having a higher Ca/P ratio favor adsorption of negatively charged alendronate compared with the HAn nanoparticles.

Figure 8. Released mass percentages normalized with respect to the HA surface areas of cisplatin (CDDP, circles), DPM (squares), and alendronate (triangles) as a function of time for plate-shaped (full symbols) and needle-shaped (empty symbols) HA nanoparticles. Data represent mean values divided by a normalization constant; error bars indicate the associated standard deviations.
This effect, however, is evident only at high concentrations, where the HA binding sites are probably saturated. On the other hand, HAn nanoparticles having a lower Ca/P ratio slightly favor adsorption of positively charged species of hydrolyzed cisplatin. With regard to the release kinetics, neutral DPM is released in preference to positively charged aqutated cisplatin and negatively charged alendronate. It is possible that short range electrostatic interactions between aqutated cisplatin and surface phosphates, and complexation between anionic alendronate and surface Ca\(^{2+}\) ions, disfavor release of these drugs. Moreover, it appears that release of DPM takes place through complete cleavage of the platinum–medronate bond. Finally, whilst desorption of charged species (aqutated cisplatin or anionic alendronate) is not dependent on the type of HA nanoparticle used, the release of neutral DPM molecules is greater when adsorbed to HAn rather than for HAp nanoparticles. The above results demonstrated that HA nanocrystals and antitumor drugs can be selected in such a way that the bioactivity of the drug–HA conjugate could be tailored for specific therapeutic applications.

5. Experimental

cis-Diaminedichloridoplatinum(II) (cisplatin) was purchased from Sigma–Aldrich S.r.l, and sodium alendronate was a kind gift from Merck Sharp & Dohme, Italy. [Pt(en)(H\(_2\)O)(SO\(_4\))] \(\text{en = ethylenediamine}\) was prepared according to reported procedures [33].

Di(ethylenediamineplatinum)medronate (DMP): [Pt(en)(H\(_2\)O)(SO\(_4\))] (500 mg, 1.35 mmol) in water solution (20 mL) was treated with a suspension of barium medronate (BaH\(_2\)MDP, 421 mg, 1.35 mmol) in 20 mL of water. An immediate white opalescence indicated the instantaneous formation of barium sulfate. After ca. 30 min of stirring, the suspension was filtered to remove BaSO\(_4\), and the filtrate concentrated to half of its original volume. The precipitate was separated by filtration of the solution and dried. Anal. Found: C, 8.64; H, 2.68; N, 7.81. 1HN M R(\(\text{D}_2\)O): \(2.05 \text{ ppm} (2H, t, 2\Delta h = 2.88; H, 2.88; N, 8.00). \text{CDCl}_3, 2180–2188 © 2007 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim www.afm-journal.de 2187

\[ L_{(0,2)} = \frac{0.944 \cos \theta (\Delta L^2 - A_0^2)}{C_{113}} \]  

where \(\theta\) is the diffraction angle for plane (002), \(\Delta r\) and \(\Delta \delta\) the widths in radians of reflection (002) at half height for the synthesized and the reference HA materials, respectively, and \(\lambda = 1.5405 \text{ Å}\). The degree of HA crystallinity was evaluated according to the formula

\[ \text{crystallinity} = (X/Y) \times 100 \]  

where \(X\) = net area of diffracted peaks, and \(Y\) = net area of diffracted peaks + background area [31].

Specific surface area measurements were undertaken using a Carlo Erba Sorpty 1750 instrument by measuring N\(_2\) adsorption at 77 K and adopting the well-known Brunauer–Emmett–Teller (BET) procedure [37]. Calcium, platinum and phosphorous contents were measured using inductively coupled plasma (ICP) optical emission spectrometry (OES) using a Perkin-Elmer Optima 4200 DV. The samples were initially dissolved in ultrapure nitric acid 1 \% to obtain elemental concentrations of between 1 and 8 ppm. Solution concentrations of cisplatin and DPM were determined using UV-vis spectrophotometric analysis. The UV-vis spectra were recorded between 190 and 300 nm using a Varian Cary 5 UV-vis-nearIR spectrophotometer, and the absorbance was measured at 205 and 230 nm against reagent blanks for cisplatin and DPM, respectively. Alendronate concentrations were measured by a colorimetric method based on the reaction of the primary amino group with ninhydrin in methanolic medium in the presence of 0.05 mol L\(^{-1}\) sodium bicarbonate. The colored product was measured at 568 nm against the reagent blank [38]. Phosphorous contents were determined spectrophotometrically as molybdovanadophosphoric acid using 1 cm quartz cell [39].

X-ray photoemission spectroscopy (XPS) analyses were performed in an M-Probe Instrument (SSI) equipped with a monochromatic AlK\(_\alpha\) source (1486.6 eV) with a spot size of 200 × 750 \(\mu\)m and pass energy of 25 eV, providing a resolution for 0.74 eV. With a monochromatic source, an electron flood gun was used to compensate the build up of positive charge on the insulator samples during the analyses. 10 eV electrons were selected to perform measurements on these samples. The accuracy of the reported binding energies (BE) was estimated to be ± 0.2 eV. The quantitative data were also accurately checked and reproduced several times (at least ten times for each sample) and the percentage error was estimated to be ± 1 \%.

Statistics analysis was as follows: Determination of HA crystalite domain size along the c direction, bulk and surface Ca/P ratio, and specific surface area were carried out five times on the same synthesis product. Data are presented as mean value ± SD. Adsorption and release experiments were performed in triplicate, and results are plotted as mean values ± SD. Data obtained from both HA characterization and adsorption and release experiments were compared by a two tailed t-test. Differences were considered statistically significant at a significance level of 90 \%.

Adsorption of Drug Molecules onto HA Nanocrystals: An aliquot (1.5 mL) of cisplatin, DPM or alendronate dissolved in ultrapure water

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(10⁻³ M, 0.4 mg mL⁻¹ cisplatin, 0.8 mg mL⁻¹ DPM, 0.4 mg mL⁻¹ alendronate; cisplatin and DPM solutions required sonication for 15 and 30 min, respectively) was added to 10 mg of HA nanocrystals in a conical polyethylene Eppendorf tube having a capacity of 2 mL. After 15 s of treatment in a vortex apparatus, the HA suspension was maintained in a bincule bath at 37°C. Adsorption profiles for the platinum complexes and alendronate were determined by measuring the concentration of drug remaining in the supernatant solution with time. At scheduled times, aliquots (100 μL) of the supernatant that was well separated from the solid phase by 3 min centrifugation at 11 000 rpm on a Micro Centrifuge 4214 were removed for drug quantification and replaced with fresh water. The concentrations of cisplatin and DPM were determined by spectrophotometric and platinum ICP-OES analysis; alendronate concentrations were determined by colorimetric method described above.

**Drug Release from HA Nanocrystals:** The solid apatite–drug conjugates were washed twice with ultrapure water and freeze-dried for 48 h. A quantity (5 mg) of the bioconjugate was mixed in a polyethylene tube with 10 mL of 10 mM HEPES buffer saline (containing chloride ion concentration in the range 0.18–0.20 M). After 15 s of treatment in a vortex apparatus, the HA suspension was maintained in a bincule bath at 37°C and the amount of drug released from the HA nanocrystals was determined by measuring the platinum or alendronate concentrations in the supernatant solution with time as described above.

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